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Effect of Inactivation of the Global Oxidative Stress Regulator *oxyR* on the Colonization Ability of *Escherichia coli* O1:K1:H7 in a Mouse Model of Ascending Urinary Tract Infection

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To survive within the host urinary tract, *Escherichia coli* strains that cause urinary tract infection (UTI) presumably must overcome powerful oxidant stresses, including the oxygen-dependent killing mechanisms of neutrophils. Accordingly, we assessed the global oxygen stress regulator OxyR of *Escherichia coli* as a possible virulence factor in UTI by determining the impact of *oxyR* inactivation on experimental urovirulence in CBA/J and C57BL (both wild-type and $p47^{phox-/-}$) mice. The *oxyR* and *oxyS* genes of wild-type *E. coli* strain Ec1a (O1:K1:H7) were replaced with a kanamycin resistance cassette to produce an *oxyRS* mutant. During in vitro growth in broth or human urine, the *oxyRS* mutant exhibited the same log-phase growth rate (broth) and plateau density (broth and urine) as Ec1a, despite its prolonged lag phase (broth) or initial decrease in concentration (urine). The mutant, and *oxyRS* mutants of other wild-type ExPEC strains, exhibited significantly increased in vitro susceptibility to inhibition by H_2O_2, which, like the altered growth kinetics observed with *oxyRS* inactivation, were reversed by restoration of *oxyR* on a multiple-copy-number plasmid. In CBA/J mice, Ec1a significantly outcompeted its *oxyRS* mutant (by >1 $\log_{10}$) in urine, bladder, and kidney cultures harvested 48 h after perurethral inoculation of mice, whereas an *oxyR*-complemented mutant exhibited equal or greater colonizing ability than that of the parent. Although C57BL mice were less susceptible to experimental UTI than CBA/J mice, wild-type and $p47^{phox-/-}$ C57BL mice were similarly susceptible, and the *oxyR* mutant of Ec1a was similarly attenuated in C57BL mice, regardless of the $p47^{phox}$ genotype, as in CBA/J mice. Within the *E. coli* Reference collection, 94% of strains were positive for *oxyR*. These findings fulfill the second and third of Koch's molecular postulates for *oxyR* as a candidate virulence-facilitating factor in *E. coli* and indicate that *oxyR* is a broadly prevalent potential target for future preventive interventions against UTI due to *E. coli*. They also suggest that neutrophil phagocyte oxidase is not critical for defense against *E. coli* UTI and that the major oxidative stresses against which OxyR protects *E. coli* within the host milieu are not phagocyte derived.

Extraintestinal infections due to *Escherichia coli* constitute an enormous health problem, accounting for millions of infection episodes, billions of dollars of direct health care costs, and an estimated 40,000 deaths from sepsis annually in the United States alone (42). The urinary tract is the single most common site of *E. coli* infection, and *E. coli* is the most common cause of urinary tract infection (UTI). However, *E. coli* can infect almost any anatomical site; it is also a prominent cause of neonatal meningitis, ascending cholangitis, spontaneous bacterial peritonitis, and nosocomial pneumonia (42).

Most extraintestinal *E. coli* infections are due to specialized *E. coli* strains, termed extraintestinal pathogenic *E. coli* (ExPEC), that possess the requisite virulence properties to overcome host defenses, injure and/or invade host tissues, and incite a noxious inflammatory response (17, 21). Particularly in view of the increasing prevalence of antimicrobial resistance in *E. coli* (9, 27, 47) and the climbing incidence of *E. coli* sepsis (30, 42), nonantibiotic preventive measures are sorely needed, including possible interventions directed against *E. coli* virulence factors (25, 33).

Polymorphonuclear leukocytes (PMNs) constitute an important host defense against many acute bacterial pathogens, including *E. coli*. Within the urinary tract, contact with *E. coli* induces host uroepithelial cells to secrete interleukin-8, which recruits circulating PMNs from the vasculature into the uroepithelium and urinary space (1, 2). The PMN influx is crucial for bacterial clearance, as shown by the persistence of bacteria and absence of PMN infiltration observed in lipopolysaccharide-nonresponsive C3H/HeJ (TLR4-deficient) (36) mice following experimental bladder challenge with a urovirulent *E. coli* strain (36), in contrast to the brisk acute inflammatory response and rapid bacterial clearance observed with normal lipopolysaccharide-responsive C3H/HeN mice (10, 11, 46). The recruited PMNs phagocytose *E. coli* and undergo a respiratory burst, which generates toxic oxygen products that kill the bacteria, both directly and via secondary toxic products such as hypohalous acids (28). Similar processes presumably occur during *E. coli* infections at other anatomical sites (17, 42). Thus, to survive encounters with PMNs, ExPEC strains must defend themselves against an array of oxidative and nonoxidative antimicrobial systems.

The *E. coli* OxyR regulon comprises a variety of oxidant

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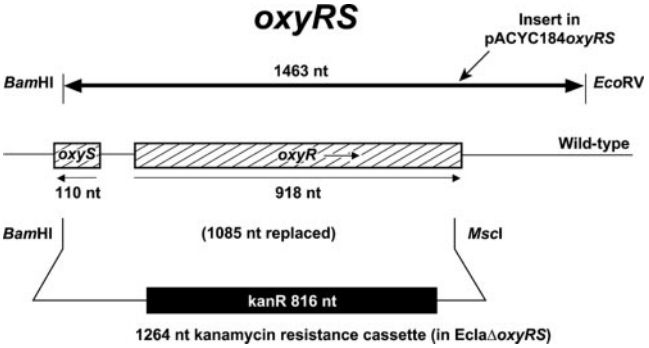


FIG. 1. Construction of Ec1aΔ*oxyRS* and pACYC184*oxyR*. Ec1aΔ*oxyRS* was created from wild-type strain Ec1a by replacing 1,085 bases in *oxyRS*, including the first 884 of 918 bases in *oxyR* and the first 103 of 110 bases in *oxyS*, with a 1,264-nucleotide (nt) kanamycin resistance cassette (bottom of figure). pACYC184*oxyR* was created by cloning into pACYC184 a 1,463-nucleotide BamH1-EcoRV genomic fragment from Ec1a that included 90% of *oxyS* and the entirety of *oxyR*, under their native promoters (top of figure).

stress response genes (31, 49). Phagocytosis of *E. coli* strain Ec1a by NADPH oxidase-competent, but not oxidase-deficient, human neutrophils increases mRNA abundance for a number of the OxyR regulon genes (44), suggesting OxyR-mediated enhancement of gene transcription in response to PMN-generated oxidants. Disruption of the chromosomal region encoding OxyR and an adjacent regulatory RNA, *oxyS*, renders *E. coli* strain Ec1a hypersusceptible to killing by PMN antimicrobial systems and has a lesser impact on bacterial killing by oxidase-deficient neutrophils (44).

Because of the critical importance of PMNs in urinary host defense (11, 46), we hypothesized that OxyR might contribute to *E. coli* urovirulence, which, if true, would make it a potential target for nonantibiotic preventive measures. Accordingly, we assessed the effect on urovirulence of *oxyR* inactivation, followed by restoration of the OxyR phenotype by complementation, in a urovirulent wild-type *E. coli* background, using an established mouse model of ascending, unobstructed UTI. Likewise, we assessed the effect of inactivation of host phagocyte oxidase (in $p47^{phox-/-}$, i.e., chronic granulomatous disease [CGD] phenotype, mice) (35) on host susceptibility to experimental UTI and on the relative virulence of a wild-type *E. coli* strain and its *oxyR* derivative.

MATERIALS AND METHODS

Bacterial strains. *E. coli* strain Ec1a is ATCC 11775, a human urinary isolate. Ec1a is serum resistant and expresses the O1 antigen (as determined by the *E. coli* Reference Center, University Park, PA). According to PCR-based virulence genotyping, it exhibits the characteristic virulence factor profile and genomic profile of the O1:K1:H7 clonal group (19, 22), including the F11 variant of *papA* (P fimbria structural subunit), allele II of *papG* (P adhesin), the K1 variant of *kpsM* (group 2 capsule), and the H7 *fliC* (flagellin) variant. According to randomly amplified polymorphic DNA analysis with arbitrary primers 1247 and OPG13 (not shown), it exhibits a genomic pattern indistinguishable from that of *E. coli* urosepsis isolates H25 (O1:K1:H7) and V23 (O2:K1:H7) but distinct from that of pyelonephritis isolate 536 (O6:K15:H31) (22, 23).

E. coli strain GS047 (i.e., MC4100Δ*oxyR*S, obtained from G. Storz [3, 48]) has a kanamycin resistance determinant (Kan^r) from Tn*903* in place of the BamHI-MscI chromosomal region encompassing *oxyR* and *oxyS* (Fig. 1). Ec1aΔ*oxyRS* was constructed by P1 transduction of Kan^r from strain GS047 into strain ATCC 11775 as previously described (44). Analysis of Ec1aΔ*oxyRS* showed it to be indistinguishable from Ec1a with respect to extended virulence genotype, XbaI pulsed-field gel electrophoresis profile, mannose-resistant hemagglutination phenotype with human erythrocytes (indicating expression of pyelonephritis-associated P fimbriae), and mannose-susceptible yeast agglutination phenotype (indicating expression of cystitis-associated type 1 fimbriae) (not shown). Plasmid pAQ5, derived from pACYC184, bearing the *oxyR* gene and 90% of the *oxyS* gene under their native promoters (Fig. 1), was obtained from G. Storz (24, 45), designated as pACYC184*oxyRS*, and transformed into EC1 by electroporation.

P1 transduction also was used to transfer Kan^r from strain GS047 into two widely studied ExPEC strains, including CP9 (O4:K10,54/96:H5; bacteremia) (39, 40) and RS218 (O18:K1:H7; neonatal meningitis) (4, 13). The resulting transductants were confirmed to contain the deletion of *oxyR*, but were otherwise unaltered, by using the procedures as described for confirmation of Ec1aΔ*oxyRS* and also were transformed with pACYC184*oxyR* by electroporation.

To assess the phylogenetic distribution of *oxyR* within *E. coli*, the 72 members of the *E. coli* Reference (ECOR) collection, which ostensibly reflect the range of genetic diversity in the species (32), were screened for *oxyR*, as were 14 representatives of *E. coli* clonal group A (CGA), including SEQ102 and UMN026 (cystitis isolates from Berkeley, CA, and Minneapolis, MN [29]) and 12 CGA pyelonephritis isolates from around the United States (18). Detection of *oxyR* was both by PCR, using internal *oxyRS* primers OxyRF6 (5′-ATTCCCACAGTT GGACCGTA-3′, bases 291 to 310) and OxyRR7 (5′-CGCGGAAGTGTGTAT CTTCA-3′, bases 662 to 641) and by dot blot hybridization, using a probe that was synthesized and digoxigenin labeled with the above primers. All testing was done at least in duplicate, with appropriate positive and negative controls.

H_2O_2 susceptibility assays. Bacterial susceptibility to inhibition by H_2O_2 was assessed by using an agar diffusion assay. Mueller-Hinton (MH) agar plates (one per strain) were streaked for confluence with each *oxyR* mutant and the corresponding parent strain. Sterile paper filter disks (6 mm) were placed on the surface of the plates at several locations each, after which 1 M H_2O_2 was spotted onto each disk in 10-μl aliquots (10 μmol per disk). After overnight incubation at 37°C, inhibition zone diameters around each H_2O_2 spot were measured. Mutants and parents were tested in parallel on the same day using the same media, reagents, and inoculation methods.

In vitro growth assays. Growth curves were determined for strains Ec1a, Ec1a/pACYC184, Ec1aΔ*oxyRS*, and Ec1aΔ*oxyRS*/pACYC184*oxyRS*. Serial dilution plating was used to assess bacterial concentrations at intervals during growth for 2 to 24 h at 37°C in shaken or static Mueller-Hinton or Luria-Bertani (LB) broth, prereduced and anaerobically sterilized brain heart infusion broth in sealed anaerobic tubes (Remel), or filter-sterilized human urine from a healthy volunteer. Chloramphenicol (20 μg/ml) was added to cultures containing pACYC184-based transformants.

Mouse model of UTI. A well-established dual-strain (competition) model of unobstructed, atraumatic ascending UTI was used to compare the urinary tract-colonizing abilities of Ec1a and Ec1aΔ*oxyRS* and of Ec1a/pACYC184 and Ec1aΔ*oxyRS*/pACYC184*oxyRS* and to assess the impact of the $p47^{phox-/-}$ genotype on UTI outcomes (15, 16, 37, 38, 41, 43). A competition model was used because this approach minimizes the impact of mouse-to-mouse variation and enhances sensitivity for detecting differences between test strains. Challenge bacteria were prepared separately by overnight growth at 37°C in shaken LB broth containing chloramphenicol (30 mg/liter for pACYC-based transformants) or no antibiotics (for Ec1a and Ec1aΔ*oxyRS*). Broths were pelleted and then resuspended in LB broth, and the two test strain suspensions were combined. The combined bacterial suspension was then infused perurethrally into anesthetized 6- to 10-week-old female mice using a Harvard infusion pump (1.0 μl per g body weight, i.e., approximately 10^9 CFU/mouse), using conditions that avoid immediate vesicoureteral reflux (16, 38). For experiments involving transformants, to maintain selective pressure on plasmids, the mouse drinking water was supplemented with chloramphenicol (100 mg/liter) beginning 24 h before inoculation.

Two days after inoculation, mice were euthanized. Aseptically harvested urine samples and bladder and kidney homogenates were cultured quantitatively. Replica plating to appropriate antibiotic-supplemented agar plates (kanamycin, 50 mg/liter; tetracycline, 12.5 mg/liter; chloramphenicol, 30 mg/liter) was used to separately enumerate the two test strains in each experiment from both the inoculum suspension (64 colonies per culture) and the postmortem cultures (32 colonies per culture). For experiments involving Ec1a and Ec1aΔ*oxyRS*, kanamycin-resistant colonies were scored as Ec1aΔ*oxyRS* and kanamycin-susceptible colonies as Ec1a. For experiments involving transformants, tetracycline-resistant, kanamycin-susceptible colonies were scored as Ec1a/pACYC184 and kanamycin-resistant, tetracycline-susceptible colonies as Ec1aΔ*oxyRS*/pACYC184*oxyR*. (Colonies from the latter experiments were also assessed for chloramphenicol susceptibility to confirm retention of plasmids.) To confirm the inferred strain identities, one putative representative of each test strain from each positive

TABLE 1. Inhibition by 10 μmol H_2O_2 of wild-type *Escherichia coli* strains, their *oxyRS* mutants, and *oxyRS*-complemented mutants

Strain	Mean inhibition zone diam[a] ± SD (mm) (*P* value versus Δ*oxyRS* mutant[b])		
	Wild type	Δ*oxyRS* mutant	Complemented mutant[c]
Ec1a	18.3 ± 1.3 (0.001)	33.3 ± 3.3	20.0 ± 1.4 (<0.001)
CP9	19.0 ± 1.4 (<0.001)	34.3 ± 3.1	19.8 ± 1.3 (<0.001)
RS218	19.0 ± 1.2 (0.001)	35.8 ± 1.7	19.3 ± 1.3 (<0.001)

[a] Mean of four determinations per strain, once daily for 4 days. All strains were tested in parallel each day.

[b] P values (by two-tailed *t* test) are for comparisons of the wild type or complemented mutant versus the Δ*oxyRS* mutant. All comparisons of the wild type versus complemented mutant yielded a *P* of >0.10.

[c] Complemented mutant was the Δ*oxyRS* mutant transformed with pACYC184-*oxyRS*.

culture was compared with the actual test strain. This was done by using randomly amplified polymorphic DNA analysis (5, 23) and assessment of *oxyR* status (by PCR, using primers OxyRF6 and OxyRR7) and H_2O_2 susceptibility (by agar diffusion, as described above). The ratio of the two test strains in postmortem mouse cultures (output ratio) was adjusted for the test strains' ratio in the inoculum suspension (input ratio) to derive the competitive index (CI) for each mouse infection culture.

Neutrophil oxidase-deficient ($p47^{phox-/-}$) mice. For the initial virulence experiments (above), CBA/J mice were used (Harlan Sprague-Dawley, Indianapolis, IN). Since $p47^{phox-/-}$ mice in the CBA/J background were not available, C57BL mice (normal and $p47^{phox-/-}$) were obtained from Jackson Laboratories (Bar Harbor, ME) (35). Experimental methods were as described above. The study design was as follows. Each week for 2 weeks, five normal and five $p47^{phox-/-}$ C57BL mice were inoculated in parallel with a suspension of Ec1a and Ec1aΔ*oxyR*. Comparisons were made of total bacterial counts in C57BL versus (previously studied) CBA/J mice, total bacterial counts in normal versus (concurrent) $p47^{phox-/-}$ C57BL mice, and relative (competitive) bacterial counts for Ec1a versus Ec1aΔ*oxyR* in C57BL mice, both overall and stratified by $p47^{phox}$ status.

Statistical methods. Comparisons of proportions were tested using Fisher's exact test (two-tailed). Comparisons involving inhibition zone diameters or absolute bacterial counts were tested using an unpaired *t* test (two-tailed), whereas those involving CIs were tested using the Wilcoxon rank sum test, with zero used as the comparator for each culture's $\log_{10}$ of the CI ($\log_{10}$ CI) value.

RESULTS

Effect of *oxyRS* knockout on the H_2O_2 susceptibility of Ec1a and other ExPEC strains. The *oxyRS* mutants of strains Ec1a, CP9, and RS218 all exhibited significantly larger zones of inhibition around filter disks impregnated with 10 μmol H_2O_2 than did the corresponding parent strains, indicating enhanced H_2O_2 susceptibility with inactivation of *oxyRS* (Table 1). Inhibition zones were restored to parental size by complementation in *trans* with an *oxyR*-expressing pACYC184 construct, confirming the specificity of the observed effect for *oxyR* (Table 1).

Growth of Ec1a and Ec1aΔ*oxyRS* and their transformants in broth and urine. During in vitro growth in broth (whether LB, MH, or anaerobic), under shaking or static conditions, Ec1aΔ*oxyRS* exhibited a 2-h to 4-h lag phase in comparison with the parent before beginning log-phase growth. Thereafter, it exhibited a maximal growth rate similar to that of the parent and ultimately achieved the same plateau density (Fig. 2, top). The mutant's growth pattern was restored to that of a parental vector control by complementation in *trans* with an *oxyR*-expressing pACYC184 construct, confirming the specificity of the effect for *oxyR* (Fig. 2, top).

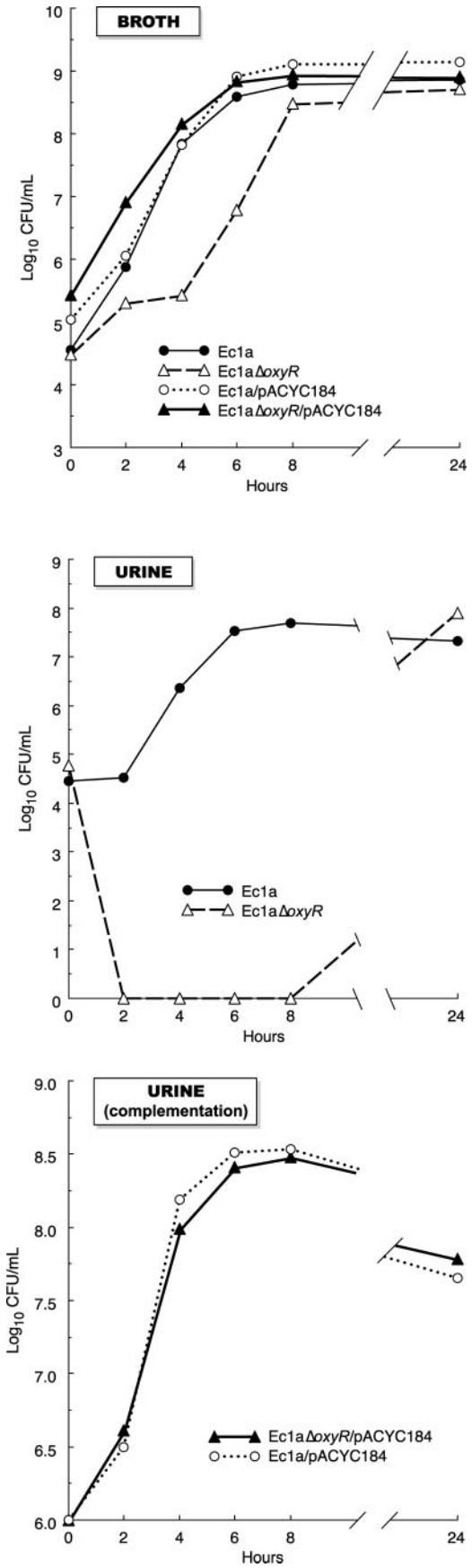
During ex vivo incubation in filter-sterilized human urine, the parent Ec1a exhibited a 2-h lag phase, followed by brisk log-phase growth (Fig. 2, middle). In contrast, after being added to urine, the *oxyRS* mutant rapidly dropped to undetectable concentrations and remained undetectable throughout the 8-h sampling period. However, by 24 h, it had regrown to the parental level (Fig. 2, middle). Complementation of the mutant with *oxyRS* rendered it resistant to this inhibitory (or bactericidal) effect of urine, resulting in a urine growth curve indistinguishable from that of the parental vector control (Fig. 2, bottom). All of these growth effects were observed reproducibly in multiple replicate experiments (not shown).

Comparative urovirulence of Ec1a and Ec1aΔ*oxyRS* in the mouse UTI model. Ec1a and its isogenic *oxyRS* mutant were administered perurethrally as a mixed inoculum to female CBA/J mice (18 to 25 g body weight) in a competition model of ascending UTI, using controlled inoculation conditions that avoid immediate vesicoureteral reflux. In urine, bladder, and kidney cultures harvested from mice 48 h after inoculation, according to analysis of the CI, the parent strain outcompeted the mutant by a median of >1.0 $\log_{10}$ in urine and bladders and >2.0 log10 in kidneys (Fig. 3, top). For bladders and both kidneys, the mutant's attenuation in colonization ability was highly statistically significant (Fig. 3). This supported the hypothesis that a functional *oxyRS* contributes to urinary tract colonization by strain Ec1a.

Complementation of Ec1aΔ*oxyRS* fulfillment of Koch's molecular postulates. To exclude the possibility that the attenuated urovirulence of Ec1aΔ*oxyRS* was due to alterations other than inactivation of *oxyRS* (e.g., polar effects or inapparent *trans* secondary mutations), an *oxyRS* transformant of Ec1aΔ*oxyRS* (i.e., Ec1aΔ*oxyRS*/pACYC184*oxyRS*) was used to compete against a parental plasmid control (i.e., Ec1a/pACYC184) in the same mouse model system, with chloramphenicol administered to the mice to maintain selective pressure for the plasmids. The colonization ability of the complemented mutant, as assessed 48 h after inoculation, equaled (bladders) or significantly exceeded (urine and both kidneys) that of the parental plasmid control (Fig. 3). This confirmed *oxyRS* as a significant contributor to experimental urovirulence in strain Ec1a.

Effect of host phagocyte oxidase inactivation. We next assessed the importance of the host-generated oxidative environment to (i) the overall host susceptibility to *E. coli* UTI and (ii) the virulence attenuation resulting from *oxyR* inactivation in strain Ec1a. Weekly for 2 weeks, 10 female C57BL mice (five wild-type and five $p47^{phox-/-}$, i.e., CGD phenotype) were challenged per urethra with a mixture of Ec1a and Ec1aΔ*oxyR*. Total bacterial concentrations were used to assess host susceptibility, whereas the CI (output ratio for Ec1a versus Ec1aΔ*oxyR*, normalized to the input ratio) was used to assess the relative recovery of the two bacterial genotypes from each culture-positive urine, bladder, and kidney specimen as harvested 48 h postinoculation.

Overall, C57BL mice exhibited a lower susceptibility to kidney infection with Ec1a than did CBA/J mice (4 of 40 kidneys culture positive for C57BL versus 32 of 80 for CBA/J; $P < 0.001$). However, among C57BL mice, the CGD phenotype was associated with no greater susceptibility to *E. coli* UTI than the normal phagocyte oxidase phenotype. That is, in the two $p47^{phox}$ genotypes ($p47^{phox-/-}$ and normal), a similar proportion of postmortem samples was culture positive (urine, 6 of 7



versus 6 of 8 in each genotype; bladder, 10 of 10 in each genotype; kidney, 2 of 20 in each genotype). Likewise, among the culture-positive samples, total bacterial counts were similar ($P > 0.10$ for each comparison between genotypes) (data not shown).

As previously observed in CBA/J mice, the *oxyR* mutant exhibited a significant colonization deficit at each site sampled (median $\log_{10}$ CI for urine, >1.35 [$P = 0.005$]; bladders, 0.79 [$P = 0.002$]; and kidneys, >1.22 [$P = 0.045$]) relative to parent strain Ec1a. The virulence attenuation associated with *oxyR* inactivation was apparent in each C57BL genotype, and to similar degrees, regardless of the phagocyte oxidase phenotype ($P > 0.10$ for urine, bladders, kidneys, and all sites combined) (data not shown).

Phylogenetic distribution of *oxyR*. Among the 72 members of the ECOR collection, *oxyR* was reproducibly detected by both PCR and probe hybridization in 63 strains (88%), only by probe in four strains (6%), only by PCR in one strain (1.4%), and by neither modality in four strains 6%). Thus, 68 (94%) of the 72 ECOR strains were *oxyR* positive according to one or both modalities. PCR negativity but probe positivity for *oxyR* (suggesting the presence of a variant version of *oxyR*) was significantly more common within group B1 (3 of 16, 19%) than among other ECOR strains (1 of 56, 2%; $P = 0.03$). Interestingly, the only non-B1 strain that exhibited this PCR-negative, blot-positive pattern was ECOR47 (group D), a pre-1980 sheep isolate from New Guinea that is a member of, or is closely related to, the recently described multidrug-resistant *E. coli* CGA (18, 20, 29). Accordingly, additional representatives of CGA were studied. All 14 (100%) recent human clinical isolates of CGA from across the United States that were tested exhibited the same PCR-negative, blot-positive *oxyR* genotype as did ECOR47 (not shown).

DISCUSSION

We found that inactivation of *oxyRS* in wild-type ExPEC strain Ec1a significantly increased its susceptibility to H_2O_2 and attenuated its virulence in a mouse model of ascending UTI in two different mouse strains that differed in their susceptibilities to kidney infection. Moreover, complementation of *oxyR* with a multiple-copy-number recombinant plasmid restored the mutant's H_2O_2 tolerance and bladder-colonizing ability to parental levels and yielded supranormal kidney col-

FIG. 2. Representative growth curves (in broth and urine) for *Escherichia coli* strains Ec1a and Ec1aΔ*oxyRS* and transformants of each. Bacterial concentrations at each time point were determined by serial dilution plating. (Top) Growth in Mueller-Hinton broth of Ec1a, Ec1aΔ*oxyRS*, Ec1a/pACYC184, and Ec1aΔ*oxyRS*/pACYC184*oxyR*. Testing of all four strains was done in parallel on the same day and was replicated on multiple days. Chloramphenicol supplementation was used with Ec1a/pACYC184 and Ec1aΔ*oxyRS*/pACYC184*oxyR*. The extended lag phase observed with Ec1aΔ*oxyRS* was highly reproducible, whereas no consistent differences were noted among the other three strains. (Middle) Growth in sterile human urine of Ec1a and Ec1aΔ*oxyRS*. The mutant was reproducibly undetectable from 2 to 4 h out to the 8-h time point but achieved parental densities by the 24-h time point. (Bottom) Growth in sterile human urine (supplemented with chloramphenicol) of Ec1a and Ec1aΔ*oxyRS*. The complemented mutant and (plasmid) control exhibit indistinguishable growth kinetics.

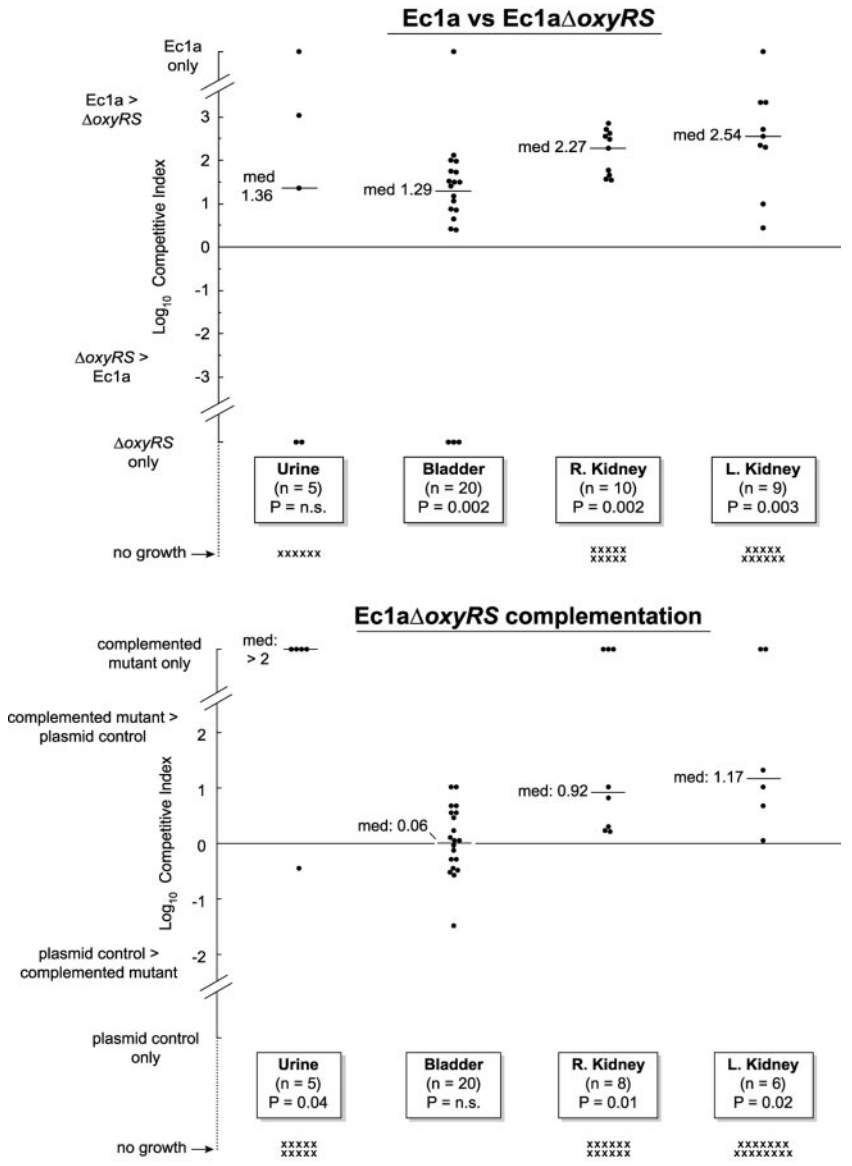


FIG. 3. Comparative mouse urinary tract colonization ability of *Escherichia coli* strains Ec1a and Ec1aΔ*oxyRS* and transformants of each. A competition model was used in which both test strains (for a given experiment) were administered simultaneously to female CBA/J mice perurethrally as a mixture. Outcomes (from 48-h postchallenge cultures of urine, bladder, and kidneys) are displayed as the $\log_{10}$ of the CI, which is the relative prevalence of the two test strains in the postmortem cultures (output ratio), corrected for the strains' relative prevalences in the challenge suspension (input ratio). Positive values for the $\log_{10}$ CI indicate a competitive advantage for Ec1a over Ec1aΔ*oxyR* (top) or for the complemented mutant (Ec1aΔ*oxyRS*/pACYC184*oxyR*) over the plasmid control (Ec1a/pACYC184) (bottom). Negative values for the $\log_{10}$ CI indicate the reverse. Horizontal lines indicate the median (med) values among positive cultures. For each experiment, 10 mice were studied each week for 2 weeks (20 mice total per experiment). Postmortem urine samples were not available from all mice. *P* values are by the Wilcoxon rank sum test. n.s., not significant.

onization. This indicates that OxyR, a global oxygen stress response regulator, facilitates urovirulence in *E. coli* strain Ec1a and, hence, could be considered a virulence-facilitating factor, if not a virulence factor per se.

OxyR's contribution to urovirulence likely derives from its protective role against oxidative stress and injury. The acute host inflammatory response to UTI includes a vigorous influx of PMNs, which, upon encountering the pathogen, generate and release reactive oxygen products and their toxic derivatives (11, 28). The OxyR system presumably enables *E. coli* to better withstand this noxious onslaught. However, even during ex vivo growth in urine from a healthy volunteer, the *oxyRS* mutant exhibited marked growth derangements in comparison with the parent and the complemented mutant; these were much greater than the subtle defects observed during in vitro growth in nutrient broth. This suggests that non-PMN-derived oxidative factors may be present even in uninfected urine. Alternatively, growth in urine may require metabolic pathways that generate substantial endogenous oxidants especially toxic to *oxyR*-deficient *E. coli*.

To directly assess the relative contribution of the oxidative killing system of PMNs, as opposed to other oxidative stresses present within the urinary tract, to the virulence deficit observed with the *oxyRS* mutant, we utilized $p47^{phox-/-}$ CGD phenotype mice, which are deficient in phagocyte oxidative killing. (The C57BL strain, although less susceptible than the more commonly employed CBA strain, was used as a comparison for the otherwise isogenic phagocyte oxidase-deficient mouse line.) OxyR mutants of *E. coli* were attenuated for virulence in C57BL mice much as they were in CBA/J mice, suggesting that oxidative stresses imposed on bacteria in the urinary tract are important for suppressing the pathogenesis of infection in at least these two strains of mice. However, our prior hypothesis that the source of oxidative suppression of bacterial virulence is primarily the phagocyte oxidase was not supported by these experiments using phagocyte oxidase-deficient mice. That is, the virulence of *oxyR E. coli* was not increased by eliminating host phagocyte oxidase activity. Surprisingly, the virulence of wild-type *E. coli* was also not increased by eliminating host phagocyte oxidase activity. Thus, although in the mouse UTI model, neutrophils are important in host defense against ascending pyelonephritis (10–12), the neutrophil phagocyte oxidase appears not to be critical. Neutrophils maintain a substantial antimicrobial armamentarium that is phagocyte oxidase independent and these are presumably the principal factors that suppress ascending pyelonephritis in this setting.

What, then, are possible sources of nonneutrophil oxidative stress that attenuate the virulence of *oxyR E. coli* and are responsible for the profound growth defect observed in human urine? Hpx^- *E. coli*, a recently coined term (34), is deficient in three enzymes that detoxify hydrogen peroxide: hydroperoxidase I (*katG* gene), hydroperoxidase II (*katE*), and alkyl hydroperoxidase (*ahpC*/*ahpF*). Such strains accumulate endogenous hydrogen peroxide in their cytosol as a consequence of aerobic metabolism and are unable to defend themselves against exogenous hydrogen peroxide. One consequence of the *hpx*-deficient genotype is a growth defect and increased susceptibility to oxidative DNA damage (34). Expression of both hydroperoxidase I and alkyl hydroperoxidase is regulated in part by OxyR. Thus, it is possible that *oxyR E. coli* acquires a partial Hpx^- phenotype even though the genes for all three enzymes are present. Conceivably, growth conditions within the urinary tract, and in urine per se, serve as a metabolic stress that impairs the growth and survival of the *oxyR* strain. Additional stresses may be engendered by the oxygen-independent antimicrobial armamentarium of urinary phagocytes. Why, however, the reactive oxygen species generated by the phagocyte oxidase do not further impair the virulence of *oxyR E. coli* remains an unanswered question.

Our experimental findings satisfy, for *oxyR*, the second and third of the molecular versions of Koch's postulates, as articulated by Falkow to describe the evidence needed to establish a particular microbial trait as a virulence factor (8). The second postulate holds that inactivation of the trait must diminish virulence, whereas the third requires that restoration of the trait must restore virulence. In contrast, the first postulate, i.e., that the trait must be epidemiologically associated with disease, cannot be fulfilled for *oxyR* or, for that matter, any property that is ubiquitous (or nearly so) within the species, even if it demonstrably contributes to virulence and is not required for vegetative growth. For *E. coli*, such traits include type 1 fimbriae, *guaA*, and *argC* (7, 26, 41).

Which specific components of the *oxyR* regulon contributed to the observed enhanced virulence remains to be determined. One or more of these components may be both functionally important and amenable to vaccine or pharmaceutical interference and thus may constitute a useful therapeutic target. Because of the nearly ubiquitous nature of OxyR, before any such interventions are implemented clinically, it would be important to confirm that they do not exert health-harmful effects on the host's commensal flora.

The supercolonization phenotype exhibited by the *oxyR*-complemented mutant was most likely due to increased gene dose, since the carrier plasmid, pACYC184, is present in approximately 18 copies per cell (6). This finding provides further suggestive evidence of the importance within the urinary tract of oxygen-mediated host defenses (28) and, correspondingly, the value to uropathogens of oxidative stress protection systems. That supercolonization was observed in kidneys but not bladders may suggest that oxygen-related host defenses are more pronounced in the kidney.

It appears that the transfer of *oxyR E. coli* to fresh medium or urine under a variety of circumstances (LB or MH broth, aerobic or anaerobic) results in the stasis or even death of many organisms soon after transfer. However, after a lag phase of 2 to 4 h (medium) or >8 h (urine), the organisms and their progeny appear to grow as rapidly as the parental strain. The growth defect can be attributed with confidence to the *oxyR* mutation, since it disappears when complemented with a normal *oxyR* gene in *trans* (multiple copy number). We suggest that the transfer to new medium generates an endogenous oxidative stress (14) that is addressed promptly, by *oxyR*-dependent mechanisms, in the parental strain and more slowly, by *oxyR*-independent mechanisms, in the mutant. We also suggest that the failure of the mutant to catch up with the parent in vivo as it does in vitro is attributable in part to the greater and ongoing oxidative stress within the (infected) host urinary tract.

The in vitro growth effects associated with *oxyRS* inactivation beg the question of whether OxyR can be considered a true virulence factor in the classical sense. Presumably, inactivation of any bacterial trait that is required for in vitro growth would both impair in vivo fitness and be complementable, thereby allowing fulfillment of the second and third of Koch's molecular postulates. Clearly, this is insufficient to consider a trait a virulence factor. However, with OxyR, we observed a gradient of growth effects, from minimal (in vitro, with broth or agar plates), to intermediate (ex vivo, in urine), to profound (in vivo, in mice). We do not consider the in vivo findings to be fully explained by the in vitro effect. Thus, growth effects associated with gene knockouts may need to be considered quantitatively, against a relevant commensal comparator, rather than categorically.

In this regard, the impact of *oxyR* inactivation on fitness within the primary commensal niche for *E. coli*, the colonic lumen, is undefined. In view of the known anaerobic nature of this environment, with its predominantly (obligate) anaerobic microflora, there may be little or no need for OxyR-mediated defenses in the gut. If not, and since extraintestinal *E. coli* infections usually are not advantageous to the pathogen, it is

conceivable that the selective advantage accounting evolutionarily for the retention of OxyR within *E. coli* is enhanced survival outside the host, within the (aerobic) external environment. This would facilitate dissemination to new hosts, e.g., via the food supply, thereby promoting clonal persistence and expansion, independent of any direct effect on host colonization.

In summary, we found that the global oxygen stress response regulator OxyR functions as an urovirulence-facilitating factor in *E. coli*, promoting acute bladder and kidney colonization by strain Ec1a (O1:K1:H7) in an experimental model of UTI in both CBA/J and C57BL mice. This indicates that oxidative stress is an important aspect of host defense against UTI and that the ability to resist this stress is required for full urovirulence even by an *E. coli* strain equipped with multiple other urovirulence factors. However, since the inactivation of phagocyte oxidase did not significantly increase host susceptibility to experimental UTI or blunt the virulence attenuation of the *oxyR* mutant, PMNs probably are not a critical source of oxidative stresses for *E. coli* within the urinary tract but instead protect against pyelonephritis mainly through nonoxidative mechanisms. Since *oxyR* is present in nearly all *E. coli* strains, these findings suggest the possibility of broadly active therapeutics directed toward OxyR and/or components of its regulon, provided that such interventions do not exert health-harming effects on the commensal flora.

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